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RESEARCH ARTICLE

Glucose-regulated protein 78 mediates the anticancer efficacy of shikonin in hormone-refractory prostate cancer cells

Li-Jen Kuo · Chien-Yu Huang · Wan-Li Cheng · Chin-Sheng Hung · Chun-Te Wu · Feng-Yen Lin · Yu-Jia Chang · Ming-Te Huang

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Abstract Glucose-regulated protein 78 (GRP78) is a key modulator of prostate cancer progression and therapeutic resistance. Prostate cancer is a worldwide health problem, and therapeutic resistance is a critical obstacle for the treatment of hormone-refractory prostate cancer patients. Shikonin inhibits prostate cancer proliferation and metastasis. However, the role of GRP78 in the cytotoxic effect of shikonin in prostate cancer cells remains unclear. GRP78 expression was abolished using small interfering RNA (siRNA), and the anticancer effects of shikonin were assessed using MTT assays, the XCELLigence biosensor, flow cytometric cell cycle analysis, and Annexin V-PI apoptotic assays. PC-3 cells expressed more GRP78 than DU-145 cells, and the MTT assays revealed that DU-145 cells were more sensitive to shikonin than PC-3 cells. GRP78 knockdown (GRP78KD) PC-3 cells were more sensitive to shikonin treatment than scrambled siRNA control cells. Based on cell cycle analysis and AnnexinV-PI apoptotic assays, apoptosis dramatically increased in GRP78KD cells compared with the control PC-3 in response to shikonin. Finally, in response to shikonin treatment, Mcl-1 and Bcl-2 levels increased in the scrambled control cells treated with shikonin, whereas Bcl-2 decreased and Mcl-1 slightly increased in the GRP78KD PC-3 cells. The levels of Bax and Bad did not change in the scrambled control or GRP78KD cells after shikonin treatment. These results are consistent with the increased sensitivity to shikonin after knockdown of GRP78. GRP78 expression may determine the therapeutic efficacy of shikonin against prostate cancer cells.

Keywords Shikonin · Prostate cancer · Anti-cancer · GRP78

Introduction

Prostate cancer is the most frequently diagnosed male malignancy and the third leading cause of cancer-related death in

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men [1]. Approximately, 660,000 men worldwide are diagnosed with prostate cancer each year [1]. Androgen receptor (AR) signaling is an important survival pathway for prostate cancer, and androgen deprivation therapy (ADT) remains a mainstay treatment for advanced prostate cancer. ADT results in remission for 80–90 % of patients with locally advanced and metastatic disease [2]. The clinical response to ADT is manifested by a decline in serum prostate-specific antigen (PSA) and the relief of bone pain [3]. However, after an average of 2–3 years, the cancer progresses despite the fact that the serum testosterone level drops below 50 ng/dl. This type of cancer is termed hormone-refractory prostate cancer (HRPC). The mean survival time of metastatic hormone-refractory prostate cancer (mHRPC) is only 16–18 months [4]. Several possible mechanisms have been proposed to underlie the development of HRPC, including increasing androgen production by the adrenal glands and prostate tumor cells, the amplification of CYP17, elevated AR gene copies with high expression, variant AR splicing, and tumor escape via the glucocorticoid receptor [2, 5, 6]. Signaling cross-talk with the PI3K/AKT/ERK/mTOR family may also play a role as a cofactor [7]. However, there is currently no effective therapeutic solution for HRPC.

Shikonin, an important component of Lithospermum erythrorhizon, displays antioxidant, anti-inflammatory, antithrombotic, antiviral, antimicrobial, and anticancer effects [8–11]. Several mechanisms are involved in the anticancer activity of shikonin. Shikonin inhibits prostate cancer cell migration and promotes autophagy in vitro by inhibiting the Akt signaling pathway [12, 13]. Shikonin also acts as a tubulin inhibitor to arrest hepatoma cell growth in the G2/M phases [14]. Thus, while the precise mechanisms by which shikonin impedes cancer cell growth remain to be elucidated, shikonin-induced apoptosis has been thoroughly described [15, 16]. Interestingly, shikonin can markedly decrease the expression of AR and PSA, as well as inhibit the growth of AR-positive human prostate cancer cells [17]. Therefore, shikonin is a strong anticancer drug candidate for chemoprevention and the treatment of hormonal-responsive prostate tumors. However, the anti-cancer effect of shikonin in HRPC remains unclear.

GRP78 is a well-known endoplasmic reticulum (ER) stress response protein that plays an essential role in embryo development and cancer progression. GRP78 is highly expressed in cancer tissues [18, 19]. Targeting GRP78 suppressed proliferation and metastasis in HRPC cells [20], indicating that GRP78 may play a key role in maintaining malignancy in HRPC. However, there is currently no information regarding the role GRP78 in the anti-cancer effect of shikonin in HRPC. Here, we demonstrate that GRP78 may mediate the cytotoxic efficacy of shikonin in HRPC.

Materials and methods

Chemicals, antibodies, and cell culture

The following items were obtained from Sigma-Aldrich (St. Louis, MO, USA): shikonin, propidium iodide (PI), Tris–HCl, trypan blue, ethylenediaminetetraacetic acid (EDTA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ribonuclease A, and dimethyl sulfoxide (DMSO). GRP78, Mcl-1, and GAPDH antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Bcl-2 and Bax antibodies were purchased from Cell Signaling Technology (Boston, MA). The Bad antibody was purchased from GeneTex Inc. (Irvine, CA.). Prostate cancer cell lines (PC-3 and DU-145 cell) were purchased from the American Type Culture Collection (ATCC) and grown in RPMI Media 1640 (Life Technologies, Grand Island, NY) supplemented with 10 % (*v*/*v*) fetal calf serum in a humidified incubator at 37 °C and 5 % CO_2.

Silencing GRP-78 in PC-3 cells

The expression of GRP78 was abolished in PC-3 cells using small interfering RNA (siRNA), as previously described [21–23]. The target sequence for human GRP78 mRNA was 5′-AAGGTTACCCATGCAGTTGTT-3,′ and the scrambled siRNA sequence was 5′-AAGGTGGTTGTTTTGTTCACT-3′. GRP78 and scrambled siRNA were inserted into the pSUPERIOR vector and transfected into the cells; the stably transfected cells were selected with antibiotics, as previously described [24–26]. Briefly, 1×10^5 cells were washed twice with PBS and mixed with 0.5 μg of plasmid. One pulse was applied for 20 ms at a fixed voltage of 1.4 kV on a Neon pipette-type microporator (Invitrogen Life Technologies, Grand Island, NY) [27]. The stably transfected cells were selected using antibiotics.

MTT assay

Cells (2×10^4) were seeded into 24-well plates, incubated overnight, and various concentrations of shikonin (0-4 μM) or vehicle (DMSO) were added to the cells. The medium was aspirated at specific times, and the cells were incubated in 0.25 mg/ml MTT for 1 h. Formazan was solubilized with DMSO, and the optical density was measured at 550 nm using a spectrophotometer (GE Healthcare, Piscataway, NJ) [28].

Cell proliferation assay using the x'Celligence biosensor system

Experiments from a previous study were modified and performed using an RTCA DP instrument (ACEA Biosciences, Inc. San Diego, CA). Growth curves were constructed using 16-well plates (E-plate 16, ACEA Biosciences, Inc. San

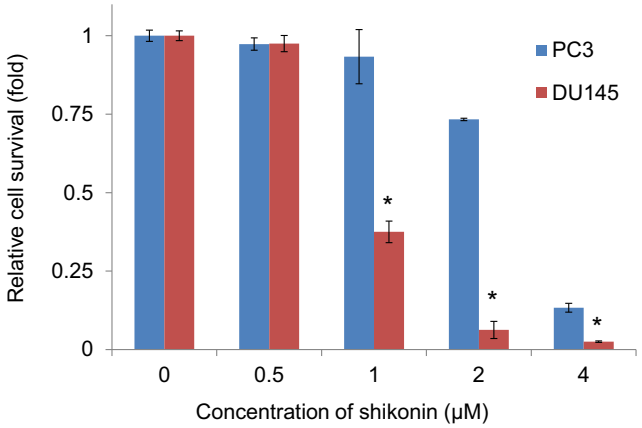

Fig. 1 The anti-proliferative effect of shikonin in prostate cancer cells. The cytotoxic effect of shikonin in prostate cancer cells (PC-3 and DU-145 cells) was determined using MTT assays. PC-3 and DU-145 cells were treated with various doses of shikonin (0–4 μM) for 48 h. Cell survival was determined using MTT assays. All of the experiments were repeated at least three times. *$p<0.05$

Diego, CA). Five thousand cells per well were seeded in an E-plate 16 in medium containing FCS and incubated with or without shikonin. The plate was monitored once every 30 s for 4 h and once every half hour thereafter. Data analysis was conducted using the RTCA software, version 1.2.

Flow cytometry for cell cycle analysis

Cells (3×10^5) were seeded into 6-well plates overnight and incubated with shikonin or vehicle for 48 h. At specific intervals, the cells were harvested, washed with PBS, and fixed in 75 % ethanol. After fixation, the cells were treated with RNase A (at a final concentration of 40 μg/ml) and stained with PI (40 μg/ml) for 30 min at room temperature. The stained cells were analyzed using Attune® Acoustic Focusing Cytometer (Life Technologies, Grand Island, NY), and the DNA content was quantified using FlowJo software (FLOWJO, LLC, Ashland, OR). The percentage of hypodiploid (sub-G_1) cells was used to quantify the dead cells.

Annexin V-PI apoptosis detection assay

Cells (5×10^5) were seeded into 6-well plates overnight and treated with shikonin or vehicle for 48 h. The cells were then harvested and washed with PBS. Next, the staining solution was prepared by diluting 2 μl of Annexin-fluorescein isothiocyanate (FITC) in 100-μl binding buffer, followed by the addition of 2 μl PI.

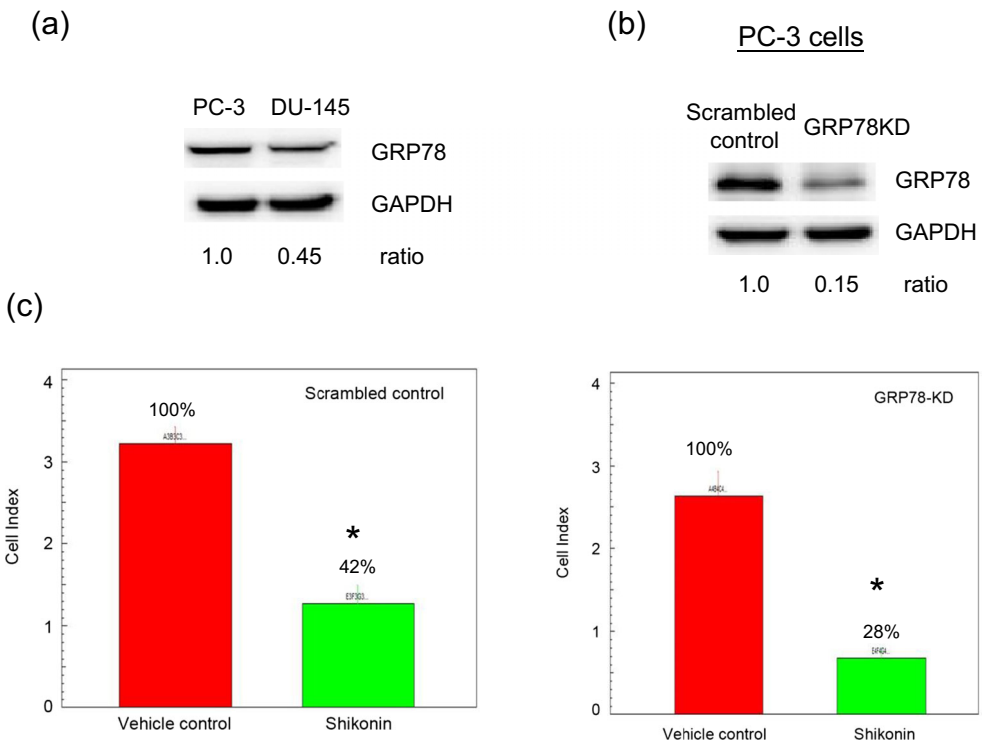

Fig. 2 GRP78 expression is correlated with the anti-proliferative effect of shikonin in prostate cancer cells. The level of endogenous GRP78 was measured and manipulated in prostate cancer cells. **a** The expression level of GRP78 in PC-3 and DU-145 cells was determined via Western blotting. **b** GRP78-siRNA and scrambled control siRNA were introduced into PC-3 cells as described in the "Materials and methods" section, and GRP78 expression levels in the scrambled control and GRP78KD cells was determined via Western blot analysis. **c** The anti-proliferative effect of shikonin in scrambled control cells and GRP78KD PC-3 cells was determined using the x'CELLigence biosensor system. The cells were incubated with 3 μM shikonin. Proliferation was monitored in real time, as described in the "Materials and methods" section. The *y*-axis represents the cell index. All of the experiments were independently repeated at least three times. *$p<0.05$

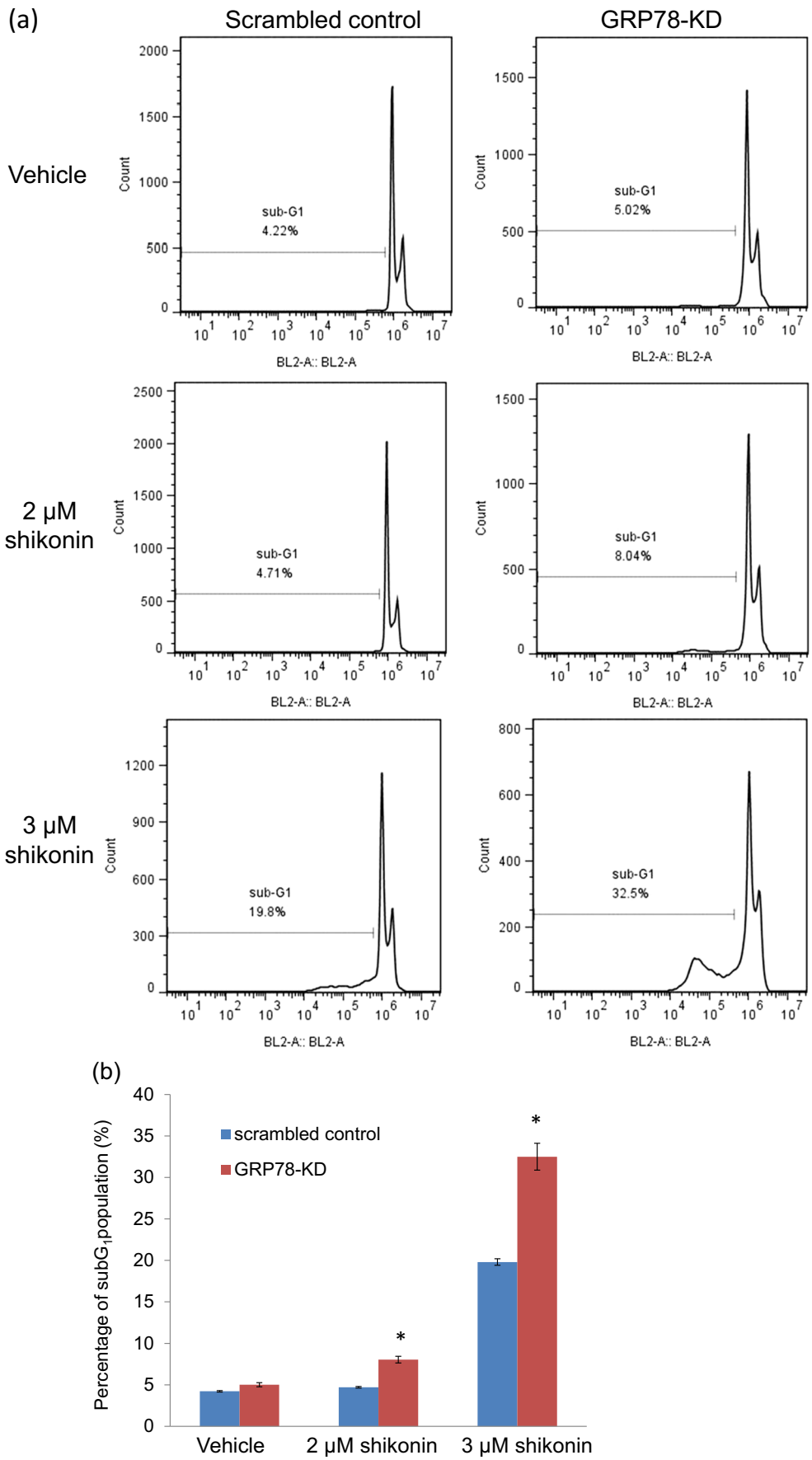

Fig. 3 Cell cycle distribution of scrambled control and GRP78KD PC-3 cells after treatment with shikonin. Scrambled control and GRP78KD PC-3 cells were exposed to 0–3 μM shikonin for 48 h. The cells were then harvested and stained with PI, and the cell cycle distribution of each sample was analyzed using a flow cytometer. **b** The $subG_1$ cell population represented apoptotic cells. All of the experiments were independently repeated at least three times. *$p<0.05$

The Annexin V-FITC kit uses a FITC-conjugated Annexin V in concert with PI (a nucleic acid dye). As the cell membrane becomes more permeable during the later stages of apoptosis, PI readily moves across the cell membrane and binds to the DNA. An Annexin V-FITC Apoptosis Detection Kit (Immunochemistry Technologies, USA) was used for the fluorescent detection of Annexin V-bound apoptotic cells as well as quantitative analysis via Attune® Acoustic Focusing Cytometer (Life Technologies, Grand Island, NY).

Western blot analysis

Control and treated cell lysates were separated using 10 % SDS-PAGE under reducing conditions and were electrotransferred onto PVDF membranes (GE HealthCare, Piscataway, NJ). The membranes were incubated with anti-GRP78, Bcl-2, Bax, Bad, Mcl-1, or GAPDH antibodies as previously described, followed by horseradish-peroxidase-conjugated secondary antibodies (1:5,000). The signals were then detected using an enhanced chemiluminescence reagent

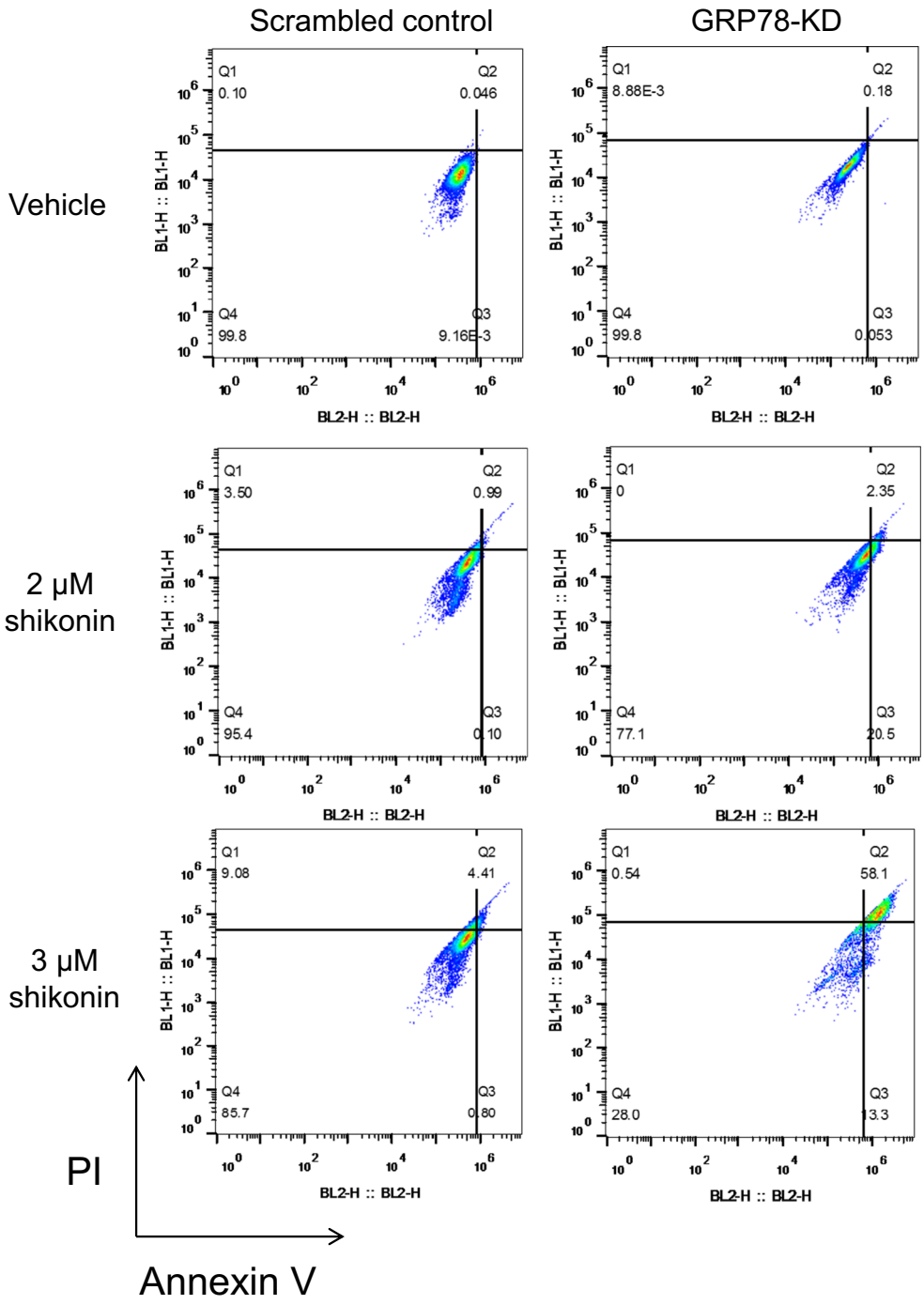

Fig. 4 PC-3 cells treated with shikonin were evaluated for evidence of apoptosis using an Annexin V-PI apoptotic assay. Scrambled control and GRP78KD PC-3 cells were treated with shikonin (0–3 μM) for 48 h, an Annexin V-FITC Apoptosis Detection Kit was used to monitor apoptosis (according to the manufacturer's instructions), and a quantitative analysis was performed via flow cytometry. The Annexin V-FITC kit uses FITC-conjugated Annexin V in conjunction with PI, a nucleic acid dye. All experiments were repeated at least three times

(GE HealthCare, Piscataway, NJ) and visualized using a VersaDoc 5000 (Bio-Rad Laboratories, Hercules, CA) [29].

Statistical analysis

All experiments were repeated at least three times. All of the data are presented as the means±standard deviation (SD). Statistical significance was determined using Student's *t* test (two-tailed) to compare two datasets. A value of $p<0.05$ was considered statistically significant.

Results

Shikonin-mediated inhibition of prostate cancer cell growth

To quantify this activity in prostate cancer cells, PC-3 and DU-145 cells were incubated with different concentrations of shikonin, and cell viability was determined using an MTT assay. Shikonin displayed dose-dependent cytotoxic effects (Fig. 1), and PC-3 cells were more resistant to shikonin treatment than DU-145 cells.

GRP78 expression levels are correlated with the antiproliferation effect of shikonin in prostate cancer cells

Consistent with our previous finding, GRP78 expression was higher in the PC-3 cells than DU-145 cells (Fig. 2a) [20]. The cells with lower GRP78 expression levels were more sensitive to shikonin treatment. To confirm the role of GRP78 in the antiproliferation effects of shikonin against prostate cancer cells, we generated GRP78 knockdown (GRP78KD) PC-3 cells in cells using silencing RNA (siRNA). As shown in Fig. 2b, the level of GRP78 expression was reduced by 85 % in the GRP78KD cells compared with the control cells. To further verify the influence of GRP78 on the antiproliferative effect of shikonin, we treated control and GRP78KD cells with shikonin. As shown in Fig. 2c, the scrambled control cells showed approximately 60 % reduced proliferative activity after shikonin treatment. In the GRP78KD cells, only 28 % of the former proliferative activity remained following shikonin treatment. These results indicate that the GRP78KD PC-3 cells were more sensitive to shikonin treatment than the control cells.

Silencing of GRP78 increases shikonin-induced apoptosis

Scrambled control and GRP78KD PC-3 cells were treated with different concentrations of shikonin (0–3 μM) or vehicle for 48 h. The cells were then harvested, fixed, and subjected to flow cytometric analyses to determine the cell cycle distribution and apoptosis. As shown in Fig. 3, a similar cell cycle distribution was observed for the scrambled control and GRP78KD cells following vehicle treatment; however, after shikonin treatment, the percentage of sub-G1 cells increased in a dose-dependent manner in the GRP78KD PC-3 cells. These data indicate that silencing GRP78 enhances the sensitivity of prostate cancer cells to shikonin treatment.

Annexin V-PI apoptosis assays

To further support our previous findings, PI and Annexin V double staining was performed to detect apoptosis. As shown in Fig. 4, the number of Annexin V-positive cells increased in the GRP78KD PC-3 cells in a dose-dependent manner after shikonin treatment. In the scrambled control cells, 3 μM shikonin induced 14 % cell death, whereas approximately 60 % cell death was observed in the GRP78KD PC-3 cells. Thus, the GRP78KD PC-3 cells were more sensitive to shikonin treatment than the control PC-3 cells.

Shikonin treatment influences the Mcl-1, Bcl-2, Bad, and Bax expression patterns in GRP78KD PC-3 cells

To characterize the molecular mechanism by which GRP78 alters shikonin-induced apoptosis in PC-3 cells, we examined the expressions of apoptosis-associated proteins in response to shikonin. Scrambled control and GRP78KD PC-3 cells were treated with shikonin for 48 h, and the expression levels of Mcl-1, Bcl-2, Bax, and Bad were detected by Western blotting. As shown in Fig. 5, the Mcl-1 and Bcl-2 levels increased

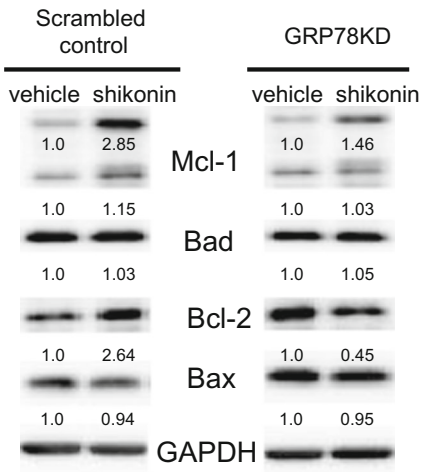

Fig. 5 Treatment with shikonin alters the expression of apoptosis-related signaling molecules in both scrambled control and GRP78KD PC-3 cells. After a 48-h treatment with shikonin (3 μM), proteins were extracted from the scrambled control and GRP78KD PC-3 cells. **a** The expression levels of Mcl-l, Bcl-2, Bax, Bad, and GAPDH were determined by Western blot analysis. GAPDH was used as an internal control. Signal intensity was calculated using a densitometer, and the relative expression levels were represented by the ratio of target protein/GAPDH. All of the experiments were repeated at least three times

in the scrambled control cells treated with shikonin, whereas Bcl-2 decreased and Mcl-1 slightly increased in the GRP78KD PC-3 cells. The levels of Bax and Bad did not change in the scrambled control or GRP78KD cells after shikonin treatment. These data suggest that silencing GRP78 sensitizes cells to shikonin by modulating the expression of apoptotic proteins.

Discussion

Prostate cancer is a critical worldwide health concern, particularly hormone-refractory prostate cancer (HRPC). There is currently no effective treatment strategy for HRPC. Several causes of HRPC have been described and several compounds have been developed to treat HRPC; however, improving the therapeutic efficacy of current drugs remains an important issue in HRPC.

Shikonin has demonstrated therapeutic potential for many diseases including ischemic brain injury and different types of cancer [12, 15–17, 30]. Shikonin inhibited prostate cancer cell proliferation and migration. Shikonin also reduced AR and PSA levels, as well as inhibited the growth of AR-positive human prostate cancer cells [17]. However, the anti-cancer effect of shikonin in AR-negative prostate cancer remains unclear. This study demonstrates that shikonin inhibits proliferation in AR-negative prostate cancer cells (PC-3 and DU-145). PC-3 cells were more resistant to shikonin treatment than DU-145 cells. Our previous finding demonstrated that GRP78 was highly expressed in PC-3 compared with DU-145 cells (Fig. 1). Thus, we proposed that GRP78 may mediate the cytotoxic effect of shikonin. To this end, we generated GRP78KD cells and assessed the cytotoxic effect of shikonin in scrambled control and GRP78KD PC-3 cells. GRP78KD cells were more sensitive to shikonin treatment (Fig. 2). Furthermore, PI staining and AnnexinV-PI apoptotic assays revealed that shikonin treatment induced apoptosis in GRP78KD cells (Figs. 3 and 4). Thus, silencing GRP78 enhances the anti-proliferative effect of shikonin.

Metastasis also remains an issue. Several studies demonstrated that shikonin also possesses anti-migratory ability. We previously demonstrated that low doses of shikonin could suppress migration in HCC but not in prostate cancer [9]. Additionally, shikonin treatment inhibited prostate cancer metastasis by reducing matrix metalloproteinase-2/-9 expression via the AKT/mTOR and ROS/ERK1/2 pathways [12]. These results indicate that shikonin represents a potential therapeutic agent for prostate cancer.

The antitumor effects of shikonin were suggested to involve in reactive oxygen species induction [31], proteasome activity inhibition [32], and cancer drug resistance circumvention [33]. Shikonin was reported to significantly activate Bax and suppress Bcl-2 expression, indicating that shikonin might tend to induce mitochondria-dependent apoptosis pathway [34]. However, several heat shock proteins (e.g., GRP78, HSP70, and HSP90) also be triggered by Shikonin [35]. Generally, GRPs are the suppressors of apoptosis [36]. Furthermore, the induction of HSP70 was shown to protect human keratinocytes against UVB-induced apoptosis [37]. Also, overexpression of GRP78 was documented to suppress apoptosis through releasing B cell lymphoma 2 (BCL-2) from BCL-2-interacting killer [38]. Recent evidences recommended that GRP78 knockdown could induce ER stress and facilitated apoptosis by TRAIL [39]. And the ER stress sensitizes cells to TRAIL through down-regulation of FLIP and Mcl-1 and up-regulation of TRAIL-R2 [39]. In our study, the GRP78 silencing in PC-3 cells exhibited higher levels of apoptosis upon shikonin treatment compared with control cells. Furthermore, the anti-apoptosis proteins Bcl-2 and Mcl-1 were down-regulated in GRP78-KD cells when compared with control. As a result, we provided evidence for first time that GRP78 depletion lead to increased sensitivity of prostate cancer cell to shikonin-induced apoptosis.

In conclusion, shikonin inhibits proliferation in AR-negative prostate cancer cells (PC-3 and DU-145). Furthermore, targeting GRP78 can enhance the cytotoxicity of shikonin in PC-3 cells. Moreover, GRP78 may mediate the therapeutic efficacy of shikonin against hormone-refractory prostate cancer cells.

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Conflicts of Interest None

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